8-Hydroxy-2′-Deoxyguanosine Is Increased in Epidermal Cells of Hairless Mice after Chronic Ultraviolet B Exposure

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8-Hydroxy-2′-deoxyguanosine (8-OHdG) is a mutation-prone (G:C to T:A transversion) DNA base-modified product generated by reactive oxygen species or photodynamic action. G:C to T:A transversions are observed in the *p53* and *ras* genes of UVB-induced skin cancers of mice and in squamous and basal cell carcinomas of human skin exposed to sunlight. In the current study, 8-OHdG formation was evaluated in the epidermis of hairless mice after repeated exposure to UVB, and possible mechanisms involved were studied. Exposure of hairless mice to either 3.4 [2 minimal erythema dose (MED)] or 16.8 (10 MED) kJ/m^2 of UVB three times a week for 2 wk induced a 2.5- or 6.1-fold increase, respectively, in the levels of 8-OHdG in DNA, compared to the unexposed controls. An immunohistochemical method using a monoclonal antibody specific for 8-OHdG showed stronger and more extensive staining in the nuclei of UV-irradiated epidermal cells than in those of nonirradiated cells. Western blots probed with antibodies against 4-hydroxy-2-nonenal–modified proteins confirmed the involvement of reactive oxygen species in the epidermal damage induced by chronic UVB exposure. 3-Nitro-L-tyrosine was detected in western blots in a concentration-dependent manner, suggesting that peroxynitrite derived from the reaction of nitric oxide and superoxide, both of which were probably released from inflammatory cells, was involved in modifying the DNA bases. Therefore, the formation of 8-OHdG after UVB exposure appears to be regulated by at least three pathways: photodynamic action, lipid peroxidation, and inflammation and may play a role in sunlight-induced skin carcinogenesis. ***Key words: oxidative DNA damage/lipid peroxidation/nitric oxide/carcinogenesis. J Invest Dermatol 107:733–737, 1997***

UV irradiation is known to cause DNA damage and is thought to be responsible for sunlight-induced skin cancers (Ananthaswamy and Pierceall, 1990). Previous studies suggested that pyrimidine photoproducts play a major role in mutation and carcinogenesis caused by ultraviolet light (Drobetsky *et al*, 1987; Keyse *et al*, 1989). Several reports, however, pointed out that types of mutations that are not supposed to be caused by pyrimidine photoproducts are observed in the human skin cancer of sun-exposed areas (van der Schroeff *et al*, 1990; Pierceall *et al*, 1991) and UVB-induced murine skin cancer (Nishigori *et al*, 1994). This implies that DNA damage other than the formation of pyrimidine photoproducts could be responsible for the UVB-induced mutations.

Since carotenoids, scavengers of reactive oxygen species (ROS), suppress experimentally UVB-induced skin tumors (Mathews-Roth, 1983; Mathews-Roth and Krinsky, 1985), ROS may play a role in UVB-induced skin carcinogenesis. At present, it is not clear how ROS are involved in the UVB-induced skin carcinogenesis. Targets of ROS include DNA, proteins, and lipids. Of these, DNA appears to be more important in carcinogenesis, because it is firmly established that cancer results from genetic alterations (Ruddon, 1995). ROS induces several types of DNA damage such as strand breaks, base modifications, and DNA–protein cross-links (Halliwell and Aruoma, 1993). 8-Hydroxy-2′-deoxyguanosine (8-OHdG), a DNA base-modified product (Kasai and Nishimura, 1984; Halliwell and Aruoma, 1993) generated by ROS is mutation-prone (G:C to T:A) (Shibutani *et al*, 1991) and has recently been shown to be a good marker for oxidative damage (Halliwell and Aruoma, 1993). It is established that hydroxyl radical, singlet oxygen, or direct photodynamic action can be responsible for the formation of 8-OHdG (Kasai *et al*, 1992; Halliwell and Aruoma, 1993).

ROS induces membrane lipid peroxidation as well, initiating the free radical chain reaction. In this process, aldehydes are generated as final products. Of these, 4-hydroxy-2-nonenal (HNE), an α,β-unsaturated aldehyde, produced almost exclusively from phospholipid-bound arachidonic acid, has been demonstrated to be a reliable index of ROS-induced lipid peroxidation, as is indicated by its cytopathologic effects (Esterbauer *et al*, 1991) and by immunohistochemical staining for HNE-modified proteins (Toyokuni *et al*,

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Abbreviations: HNE, 4-hydroxy-2-nonenal; 8-OHdG, 8-hydroxy-2′-deoxyguanosine, ROS, reactive oxygen species.

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1994a). Formation of 3-nitro-L-tyrosine is evidence for nitric oxide–mediated oxidative damage in chronic inflammation (Kaur and Halliwell, 1994), because nitric oxide reacts with the superoxide radical to give peroxynitrite that nitrates aromatic amino acid residues such as tyrosine. In spite of these advances in free radical studies, there is thus far a paucity of data regarding the involvement of ROS in UV-induced epidermal damage.

It was recently reported that UVB radiation induced 8-OHdG in cultured mouse keratinocytes (Beehler *et al*, 1992). In a previous study we reported the formation of 8-OHdG in the epidermis of hairless mice after exposure to a single large dose of UVB (Hattori-Nakakuki *et al*, 1994). In the current study, we evaluated the level of 8-OHdG after repeated UVB irradiation at a dose relevant to human exposures. In addition, we extended our research to elucidate the possible mechanism of 8-OHdG formation by assessing the involvement of lipid peroxidation and inflammation using specific antibodies.

MATERIALS AND METHODS

Animals Specific pathogen-free male hairless albino mice of the inbred strain HOS:Hr-1 (6 wk of age, Hoshino Experimental Animal Farm, Saitama, Japan) were housed in plastic cages and fed a basal diet (NF-2, Oriental, Tokyo, Japan) and tap water *ad libitum*. A total of 25 mice were used.

UV Source and Exposure The UV source was a bank of six Toshiba FL20SE fluorescent sun lamps emitting a UVB wavelength ranging from 280–320 nm, with a peak of 312.5 nm as previously described (Hattori-Nakakuki *et al*, 1994). Mice were placed in plastic cages; the floor was 25 cm below the lamps during UVB exposure. The average flux intensity at the cage floor measured with a UVR-305/365D digital radiometer (Tokyo Kogaku Kikai K.K., Tokyo, Japan) was 9.3 J/m^2/sec. The animals were divided into two groups of nine. Group I mice were exposed to 3.4 kJ UVB per m^2 three times a week with an interval of 48 h, whereas group II mice were exposed to 16.8 kJ UVB per m^2 three times a week with the same interval. Animals of each group were sacrificed immediately after the final UVB exposure (treatment of 1, 2, or 4 wk; n = 3). Seven animals were used as nonirradiated controls. Exposure to 3.4 or 16.8 kJ/m^2 was approximately twice or 10 times the minimal erythema dose (MED) for hairless albino mice, respectively.

DNA Extraction Mice were sacrificed by cervical dislocation, and irradiated back skin was immediately removed. Approximately 10 cm^2 of skin was used for DNA extraction. After incubation at 60°C for 30 s in an aluminum foil boat with the dermal surface fully attached to the aluminum, subcutaneous tissue and most of the dermis were scraped off from the epidermis with tweezers, and the epidermis was thoroughly minced. These procedures were done on ice. DNA was extracted from the minced epidermis as previously described (Hattori-Nakakuki *et al*, 1994). The samples were incubated with DNase-free RNase (400 μg per ml, Wako, Osaka, Japan) for 1 h at 37°C followed by chloroform/isoamyl alcohol extraction and ethanol precipitation.

Analysis of 8-OHdG The DNA (~40 μg) was digested by DNase I, alkaline phosphatase, and phosphodiesterase (Boehringer Mannheim, Tokyo, Japan) and analyzed by high performance liquid chromatography and electrochemical detector as previously described (Hattori-Nakakuki *et al*, 1994).

Antibodies A polyclonal antibody against HNE-modified proteins (Uchida *et al*, 1993) was used as well as the following monoclonal antibodies (MoAbs): HNEJ-2 specific for HNE-histidine adduct (Toyokuni *et al*, 1995a), N45.1 specific for 8-OHdG (Osawa *et al*, 1995), TDM-2 specific for cyclobutane pyrimidine dimers, and 64M-2 specific for pyrimidine-pyrimidone (6–4) photoproducts (Mori *et al*, 1991; Qin *et al*, 1994). A polyclonal antibody specific for 3-nitro-L-tyrosine (anti-nitrotyrosine) was obtained from Upstate Biotechnology Incorporated (Lake placid, NY).

Immunohistochemistry Skin specimens were fixed in Bouin's solution (Luna, 1968) overnight, sequentially immersed in 50% and 70% ethanol for 24 h to remove picric acid, and dehydrated. Specimens were then embedded in paraffin, sectioned at 3.5 μm, mounted on glass slides coated with poly-L-lysine, and subjected to either hematoxylin/eosin or immunohistochemical staining. In the latter, the avidin-biotin complex method (Hsu *et al*, 1981) was used to detect cyclobutane pyrimidine dimers, pyrimidine-pyrimidone photoproducts, or 8-OHdG. After 0.1% trypsin treatment and denaturing of nuclear DNA as previously described (Qin *et al*, 1994), specimens were incubated with normal rabbit serum (Dako, Kyoto, Japan; diluted to 1:75), then incubated with either MoAb TDM-2 (diluted 1:5000 in phosphate-buffered saline), MoAb 64M-2 (diluted 1:1000) or MoAb N45.1 (5 μg per ml), followed by biotin-labeled rabbit anti-mouse IgG serum (Dako; diluted 1:300), and finally avidin-biotin-alkaline phosphatase complex (Vector Laboratories, Burlingame, CA; diluted 1:100). Substrate for alkaline phosphatase (black) was obtained from Vector.

Table I. The Amount of 8-OHdG Is Increased in Epidermal Cells After Chronic UVB Exposure

Treatment Period	8-OHdG/10^5 × dG
Control (UV unexposed)	2.45 ± 0.54
Group I[a]	
1 wk	3.92 ± 0.17[c]
2 wk	6.04 ± 0.45[d]
4 wk	6.08 ± 0.47[d]
Group II[b]	
1 wk	5.12 ± 0.58[c]
2 wk	14.91 ± 2.38[d]

The amount of 8-OHdG in epidermal cells after repeated UVB exposure was measured by high performance liquid chromatography and electrochemical detector. Group I received 2 MED of UVB 3 times a week; group II received 10 MED of UVB 3 times a week, and the animals were sacrificed immediately after the final exposure (mean ± SEM; n = 3 except for the unexposed control: n = 7; [c] $p < 0.05$, [d] $p < 0.01$ *vs* unexposed control by unpaired t test; [a] $p < 0.005$, [b] $p < 0.0001$ by one-factor analysis of variance).

Western Blotting The minced epidermis was homogenized in sodium phosphate buffer containing 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg tosyl arginine methyl ester per ml, and 5 mM ethylenediaminetetraacetic acid. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL). After treatment with Laemmli sample buffer for 5 min at 100°C, the samples were run in duplicate on 12.5% sodium dodecyl sulfate-polyacrylamide gels. One gel was stained with Coomassie brilliant blue, and the other was used for western blotting as previously described (Toyokuni *et al*, 1995a). Briefly, proteins were transferred to polyvinylidene difluoride membranes, blocked with Block Ace (Dainihon Seiyaku, Osaka, Japan) and treated with the following antibodies: polyclonal antibody against HNE-modified proteins, MoAb HNEJ-2, or anti-nitrotyrosine. This was followed by reaction with horseradish peroxidase–conjugated goat anti-rabbit IgG (diluted 1:1000–1:2000: anti-HNE modified protein, anti-nitrotyrosine) or rabbit anti-mouse IgG (diluted 1:2000: HNEJ-2). Enhanced chemiluminescence immunoblotting detection reagents (Amersham, Buckinghamshire, England) were used for visualization by autoradiography. Mean density of the band area was measured using NIH Image (version 1.59) freeware, which is available from the Internet by file transfer protocol from zippy.nimh.nih.gov, after obtaining black and white image as PICT files by Epson GT-8000 scanner.

Statistical Analysis Statistical analyses were performed by an unpaired t test and one-factor analysis of variance.

RESULTS

8-OHdG Is Increased in UVB-Irradiated Epidermis Formation of 8-OHdG in the epidermis of hairless mice subjected to accumulative doses of UVB irradiation is shown in **Table I**. In this study, the absolute value of 8-OHdG in unexposed controls was 2.45 ± 0.54 8-OHdG/10^5 dG (mean ± SEM, n = 7). In group I, the 8-OHdG amount was 6.04 ± 0.45 (n = 3) after 2 wk of treatment and reached a plateau after a cumulative exposure of 20.4 kJ/m^2. In group II, the 8-OHdG amount was approximately 2.5 times higher than that of group I after 2 wk of exposure. The mice in group II died between 3 and 4 wk of treatment.

Increase in HNE-Modified Proteins After Chronic UVB Exposure One of the duplicate sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie brilliant blue reveals that the same amount of proteins were loaded to each lane **(Fig 1*a*)**. As shown in **Fig 1*b,c***, several proteins were detected in the UVB-irradiated epidermis by both polyclonal (anti-HNE–modified proteins) and monoclonal (HNEJ-2) antibodies. Polyclonal antibody against HNE-modified proteins reacts with both HNE–histidine and HNE–cysteine adducts (Uchida *et al*, 1993) whereas MoAb HNEJ-2 reacts specifically with HNE–histidine adduct (Toyokuni

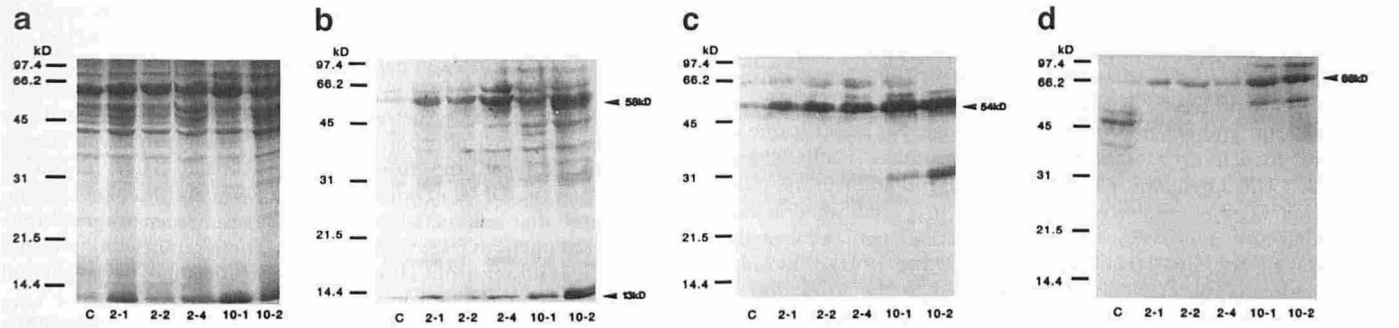

Figure 1. 4-Hydroxy-2-nonenal (HNE)-modified proteins and 3-nitro-L-tyrosine is increased in epidermal cells after chronic UVB exposure. Epidermal homogenate was prepared from the back skin of exposed or unexposed hairless mice, and 100 μg of protein in Laemmli sample buffer were loaded to each lane. *(a)* Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. Blots were probed with *(b)* polyclonal antibody specific for HNE-modified proteins; *(c)* MoAb HNEJ-2 specific for HNE-histidine adducts; *(d)* polyclonal antibody specific for 3-nitro-L-tyrosine. c, nonirradiated control skin; 2–1, 2–2, and 2–4 denote samples from group I mice (2 MED) after 1, 2, and 4 wk of UVB treatment, respectively. 10–1 and 10–2 denote samples from group II mice (10 MED) after 1 and 2 wk of treatment, respectively.

et al, 1995a). The major proteins reacting with anti-HNE–modified proteins or HNEJ-2 were approximately 58 kDa (1.99- and 2.81-fold increase after 2 wk treatment of 2 and 10 MED irradiation, respectively, by densitometry) and 13 kDa (1.53- and 3.19-fold increase, respectively) **(Fig 1*b*)** or 54 kDa (2.29- and 2.55-fold increase, respectively) **(Fig 1*c*)**, respectively. In addition, we found a dose-dependent increase in the amount of HNE-modified proteins detected after repeated UVB exposure as indicated above **(Fig 1*b,c*)**.

Increase in 3-Nitro-L-Tyrosine in Proteins After Chronic UVB Exposure Tyrosine residues of several proteins were nitrated in the UVB-irradiated epidermis **(Fig 1*d*)**. The molecular mass of the major protein was approximately 66 kDa. The amount of nitrated proteins was much higher in group II than in group I (1.97- and 3.63-fold increase after 2 wk treatment of 2 and 10 MED irradiation, respectively, by densitometry).

8-OHdG and Cyclobutane Pyrimidine Dimers Are Immunohistochemically Demonstrated Tissues treated with either phosphate-buffered saline or nonimmune mouse IgG instead of the primary antibodies showed no staining, as expected **(Fig 2*A*)**. The majority of the epidermal basal cells of untreated mice showed nuclear staining with MoAb N45.1 specific for 8-OHdG **(Fig 2*B*)**. In contrast, epidermal cells of UVB-irradiated mice showed strong and extensive staining throughout the epidermis with MoAb N45.1 **(Fig 2*C,D*)**. Some infiltrating neutrophils and macrophages, as well as fibroblasts in the dermis, showed nuclear staining. A similar pattern of staining with MoAb N45.1 was observed after UVB treatment of 2–4 wk.

To evaluate the difference in the distribution of pyrimidine photoproducts, antibodies specific for cyclobutane pyrimidine dimers (MoAb TDM-2) and pyrimidine-pyrimidone (6–4) photoproducts (MoAb 64M-2) were used. The majority of epidermal cells after UVB irradiation showed strong nuclear staining with MoAb TDM-2. Furthermore, the pattern of staining observed with this monoclonal antibody was similar to that with MoAb N45.1 **(Fig 2*E*)**. Only a few suprabasal cells, however, showed weak nuclear staining with MoAb 64M-2 **(Fig 2*F*)**.

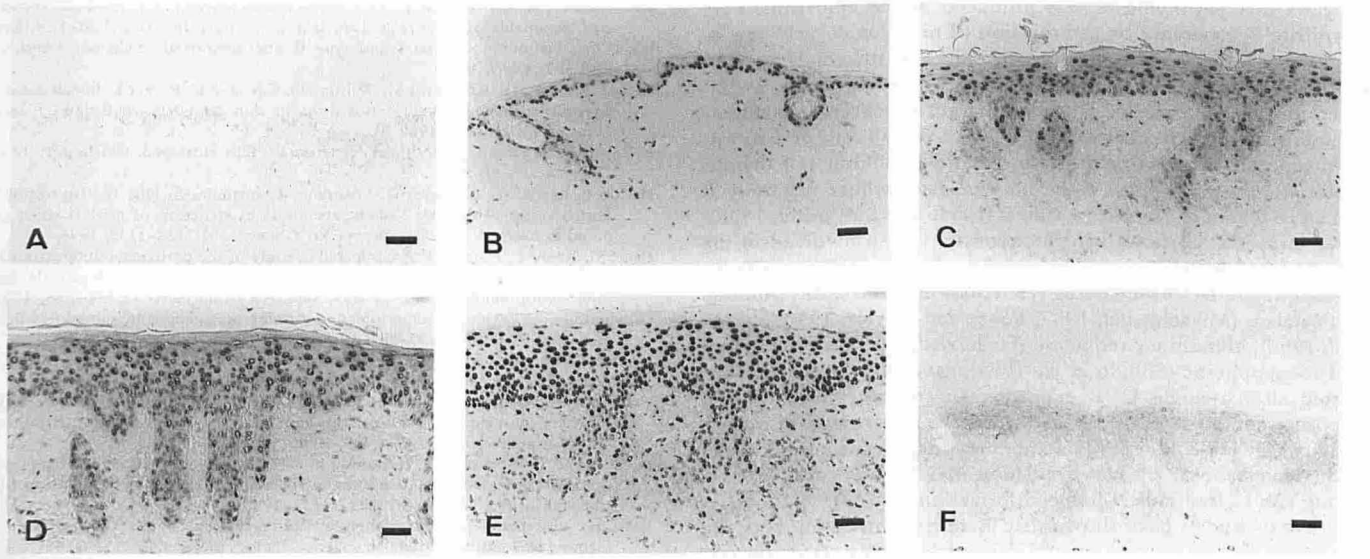

Figure 2. 8-OHdG is increased by immunohistochemistry in the nuclei of epidermis of hairless mice after chronic UVB irradiation and coexists with cyclobutane pyrimidine dimers. *(A)* Nonirradiated epidermis, no antibody; *(B)* nonirradiated epidermis stained with MoAb N45.1 specific for 8-OHdG; *(C)* epidermis from a group I mouse after 1 wk of treatment stained with MoAb N45.1; *(D)* epidermis from a group I mouse after 4 wk of treatment stained with MoAb N45.1; *(E)* epidermis from a group I mouse after 4 wk of treatment stained with MoAb TDM-2 specific for cyclobutane pyrimidine dimers; *(F)* epidermis from a group I mouse after 4 wk of treatment stained with MoAb 64M-2 specific for pyrimidine-pyrimidone (6–4) photoproducts. *Scale bar*, 100 μm.

DISCUSSION

It is known that UV radiation induces the production of ROS by photodynamic action (Foote, 1991; Kasai *et al*, 1992), thus causing several kinds of DNA damage including strand breakage, base modifications, and DNA-protein cross-linkage. 8-OHdG is one of the major oxidatively modified DNA base products (Halliwell and Aruoma, 1993; Toyokuni *et al*, 1994b) and is mutation-prone (G:C toT:A transversion) (Shibutani *et al*, 1991). It is established *in vitro* that either the hydroxyl radical or singlet oxygen induces the formation of 8-OHdG (Halliwell and Aruoma, 1993). Photodynamic action of riboflavin may be involved in the formation of 8-OHdG (Kasai *et al*, 1992; Ito *et al*, 1993). It is unknown, however, whether UV irradiation of the skin at a dose relevant to human exposure either directly or indirectly causes oxidative DNA base modification *in vivo*.

Recently, we demonstrated the formation of 8-OHdG in the epidermis of hairless mice subjected to a single large dose of UVB (101 kJ/m^2) (Hattori-Nakakuki *et al*, 1994). In the current study we investigated whether repeated UVB irradiation at a dose relevant to human exposures can induce accumulation of 8-OHdG in the epidermis and analyzed the factors that might modulate the formation of 8-OHdG. Repeated UVB exposures induced cyclobutane pyrimidine dimers and 8-OHdG simultaneously in the epidermal cells **(Table I, Fig 2*C,D,E*)**. This suggests that 8-OHdG is also one of the major modified DNA base products after UVB irradiation, although we could not compare the absolute amount in this experiment. A low level of pyrimidine-pyrimidone (6–4) photoproducts **(Fig 2*F*)** could be attributed to the induction of rapid repair activity by chronic UVB exposure.

We then assessed the involvement of ROS in epidermal damage by detecting HNE-modified proteins. The production of HNE-modified proteins was dose-dependent **(Fig 1*b,c*)**. It has previously been reported that lipid peroxidation can mediate 8-OHdG formation *in vitro* (Park and Floyd, 1992). Thus, lipid peroxidation may be another mechanism for the generation of 8-OHdG *in vivo*. The identification of proteins specifically modified by HNE is now in progress. We hypothesize that the 58-kDa and 13-kDa proteins detected by the polyclonal antibody have active cysteine residues.

Furthermore, we investigated the formation of 3-nitrotyrosine in proteins to evaluate the effect of chronic inflammation of the skin after UVB exposure. An increase in 3-nitrotyrosine **(Fig 1*d*)** suggests that peroxynitrite was produced in the epidermis. Peroxynitrite is generated by the reaction of nitric oxide with superoxide, released by infiltrating neutrophils and macrophages, and nitrates tyrosine residues of proteins (Ischiropoulos *et al*, 1992). Therefore, 3-nitrotyrosine is a good marker for chronic inflammatory states (Kaur and Halliwell, 1994). Recently Inoue and Kawanishi reported that peroxynitrite induces 8-OHdG in calf thymus DNA *in vitro* through an active intermediate whose reactivity is similar to that of the hydroxyl radical (Inoue and Kawanishi, 1995). Therefore, it is possible that peroxynitrite is also involved in the formation of 8-OHdG in epidermis after chronic UVB irradiation.

Antioxidant factors including reductions in superoxide dismutase and catalase (Miyachi *et al*, 1987; Pence and Naylor, 1990; Shindo *et al*, 1994), glutathione reductase (Fuchs *et al*, 1989), and ubiquinol and α-tocopherol (Shindo *et al*, 1994) have been previously reported after a single UVB exposure. In contrast, chronic UVB exposure for more than 12 wk raised the superoxide dismutase activity (Okada *et al*, 1994). Temporary suppression of epidermal antioxidant activity by UV irradiation may be one of the factors giving rise to free radical-induced formation of 8-OHdG.

Previous studies have shown that there is a correlation between the pyrimidine photoproducts and skin carcinogenesis (Hutchinson, 1987). Nonetheless, some mutations in UVB-induced skin cancers cannot be explained solely by the formation of pyrimidine photoproducts. Although the frequency is not high, G:C to T:A transversions in *ras* oncogenes or *p53* oncosuppressor gene are occasionally found in mouse and human skin tumors of sun-exposed areas (van der Schroeff *et al*, 1990; Pierceall *et al*, 1991; Nishigori *et al*, 1994; van Kranen *et al*, 1995; Khan *et al*, 1996). This type of transversion mutation may be attributed to the formation of 8-OHdG, as 8-OHdG can pair not only with cytosine but also with adenine, causing G:C to T:A transversions (Shibutani *et al*, 1991).

It was shown that the inflammatory response mediated by 12-O-tetradecanoyl-phorbol-13-acetate, a potent prototype tumor promoter, induced the formation of oxidized bases including 8-OHdG in the DNA of epidermal cells (Wei and Frenkel, 1991). It is of note that indomethacin, an anti-inflammatory drug, inhibits photocarcinogenesis (Reeve *et al*, 1995). These reports confirm that 8-OHdG, resulting from chronic inflammation, may be associated with UVB-induced skin carcinogenesis.

Recently, we and other investigators have found that the DNA of human cancers such as renal cell carcinoma and invasive mammary ductal carcinoma is persistently exposed to more oxidative stress than is the normal tissue, as is indicated by measurements of 8-OHdG (Okamoto *et al*, 1994; Toyokuni *et al*, 1995b). 8-OHdG is expected to play a role not only in carcinogenesis but also in tumor biology.

In summary, we have shown increased levels of 8-OHdG in the epidermis of hairless mice after repeated UVB exposure in the physiologic range. The chemistry for the formation of 8-OHdG *in vivo* appears complex and involves photodynamic action, lipid peroxidation, and inflammation. Our data suggest that formation of 8-OHdG plays a role in sunlight-induced skin carcinogenesis.

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